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SPECIAL CONTRIBUTIONS

Original Research Article

SOBERANA®Plus: safe and efficient reinforcement of preexisting natural immunity against SARS-CoV-2

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ABSTRACT

Introduction: SOBERANA Plus vaccine was evaluated in COVID-19 convalescents in the face of the emergence of new SARS-CoV-2 variants. SOBERANA Plus is based on the recombinant receptor binding domain (RBD) protein dimer with sequence 319-354. **Methods**: Two clinical studies were executed, in which one dose of the vaccine was evaluated. In the open-label Phase I clinical trial, in 30 COVID-19 convalescents with asymptomatic and mild clinical presentation, aged 22 to 57 years, safety, reactogenicity and immunogenicity were evaluated, by means of anti-RBD ELISA, in vitro RBD:hACE2 inhibition test, viral neutralization test and evaluation of effector T cells. Registered in the Cuban Public Registry of Clinical Trials, WHO-ICTRP: https://rpcec.sld.cu/en/trials/RPCEC00000349-Sp. The randomized and placebo-controlled Phase II study, in 450 COVID-19 convalescents, asymptomatic, mild or moderate, between 19 and 78 years of age. Reactogenicity and humoral immunogenicity were evaluated. WHO-ICTRP: https://rpcec.sld.cu/en/trials/RPCEC00000366-Sp. **Results and discussion:** No serious adverse events were reported in both studies. Mild events predominated, being the most common, the local pain. The vaccine stimulated an increase of >21-fold in the anti-RBD IgG antibodies 28 days after vaccination in the Phase I trial, and >31-fold in the Phase II trial. Median of inhibitory antibody titers in both phases were 94.0%. A significant increase in viral neutralizing titers against D614G, alpha, beta and delta variants, and specific IFN-*γ*- and TNF-*α*-producing T cells was detected. Conclusions, SOBERANA Plus has demonstrated an excellent safety profile and a single dose was able to effectively boost pre-existing natural immunity.

Keywords: SARS-CoV-2 Infection; COVID-19 Vaccines; safety; immunogenicity

SOBERANA®Plus: refuerzo seguro y eficaz de la inmunidad natural preexistente contra SARS-CoV-2

RESUMEN

Introducción: La vacuna SOBERANA Plus se evaluó en convalecientes de COVID-19 ante la emergencia de nuevas variantes del SARS-CoV-2. SOBERANA Plus se basa en el dímero de la proteína recombinante del dominio de unión al receptor (RBD) con secuencia 319-354. **Métodos:** Se ejecutaron 2 estudios clínicos, en los que se evaluó una dosis de la vacuna. En el ensayo Fase I abierto en 30 convalecientes de COVID-19 con cuadro clínico asintomático y leve, en edades entre 22 años hasta 57 años, se evaluó su seguridad, reactogenicidad e inmunogenicidad, mediante ELISA anti-RBD, la prueba de inhibición in vitro RBD:hACE2, la de neutralización viral y la evaluación de células T efectoras. Inscrito en el Registro Público Cubano de Ensayos Clínicos, WHO-ICTRP: https://rpcec.sld.cu/en/trials/RPCEC00000349-Sp. El estudio Fase II, controlado, aleatorizado y con grupo placebo, en 450 convalecientes de COVID-19 asintomático, leve o moderado, entre 19 años hasta 78 años de edad. Se evaluó la reactogenicidad e inmunogenicidad humoral. WHO-ICTRP: https://rpcec.sld.cu/en/trials/RPCEC00000366-Sp. **Resultados y Discusión:** En ambos estudios no se reportaron eventos adversos graves. Predominaron los leves, el más común, dolor local. La vacuna estimuló un incremento mayor que 21 veces de los anticuerpos IgG anti-RBD 28 días después de la vacunación en el ensayo Fase I, y mayor que 31 veces en el Fase II. La mediana de los títulos de anticuerpos inhibidores en ambas fases fue de 94,0 %. Se detectó un aumento significativo de los títulos de neutralización viral contra las variantes D614G, alfa, beta y delta, y de las células T específicas que producen IFN-γ y TNF-α. Conclusiones, se demostró que SOBERANA Plus tiene un excelente perfil de seguridad y que una sola dosis es capaz de reforzar eficazmente la inmunidad natural preexistente.

Palabras clave: Infección por SARS-CoV-2; vacunas contra la COVID-19; seguridad; inmunogenicidad

INTRODUCTION

COVID-19 is characterized by higher lethality in individuals with quantitative or qualitative immunity disorders and with comorbidities. The uncontrolled inflammatory response and the cytokine storm account for the torpid evolution. [1-3]

In the other clinical-epidemiological spectrum, asymptomatic infected patients are observed; also, other sick people with mild and moderate conditions, as well as individuals with evidence pointing to having suffered from a subclinical infection, detected through population serological studies. [4,5] Besides the specificities of the clinical evolution of this disease, another important characteristic is its high transmissibility. [3,5]

The percentage of asymptomatic subjects ranges from 20 % to 60 % among all cases whose PCR has tested positive. On the other hand, it has been reported that the number of infected people not detected –and hence not included in the incidence rates– may be between 10 to 20 times higher than the number of diagnosed cases, in both cases, depending on the effectiveness of the active surveillance and the health care policies established in each country. [5-7]

As regards the possibility to contract the disease again, there are diverse opinions: some researchers report immunity, in the short or long term, depending on the neutralizing antibodies. [4,8-11] Other studies report evidence of re-infection, especially in the face of new strains of SARS-CoV-2 emerging. [8,9]

In Cuba, early treatment, including the massive use of immunomodulators and anti-inflammatory drugs, does not facilitate the formation of protective antibodies and other effectors of the immune response. [6] That is why, possibly, some convalescents of COVID-19 are not adequately protected from getting re-infected by SARS-CoV-2.

The neutralizing antibodies against this virus are stimulated by S1 subunit of the spike protein, especially the domain of union with ACE2 receptor, known as RBD (receptor binding domain). Other proteins of the virus can bring the immunopathogenic mechanism mediated by antibodies: ADE (antibody dependent enhancement). [1-3,9,11] That is the reason why the development of vaccine candidates in different platforms has been based on the RBD as a thymus dependant vaccine immunogen, which have proven their safety and immunogenicity in both national and international studies. [10,12-15]

The pre-clinical evaluation of recombinant dimeric RBD (d-RBD) as the vaccine immunogen of SOBERANA®Plus has demonstrated that it is safe in toxicological and immunogenic studies in both mice and rabbits, in which high titers of antibodies against RBD have been stimulated, with a high avidity and with the capacity to inhibit the interaction of RBD with its ACE2 receptor, as well as to neutralize the living virus.

Our scientific hypothesis is based on the existence of clones of memory lymphocytes in individuals previously infected by SARS-CoV-2, who are selectively stimulated with a dose of SOBERANA®Plus vaccine, thus inducing high levels of neutralizing antibodies, which could provide protection against re-infection, especially against new variants of SARS-CoV-2. This vaccine, by reactivating in a specific way the immune response induced by the natural infection, could become a differentiated alternative for the sector of the population that has been sick with COVID-19 and was not included in the first stages of the Cuban vaccination campaign against this disease.

The general objective was to evaluate the safety, reactogenicity, and the immunogenicity of SOBERANA®Plus vaccine against SARS-CoV-2 based on d-RBD in aluminum hydroxide, in both COVID-19 convalescents whose condition was mild or moderate and those who were asymptomatic with positive PCR. The specific objectives were the following: to evaluate the safety profile of a dose of the vaccine, in both COVID-19 convalescents whose condition was mild or moderate and those who were asymptomatic with positive PCR; to evaluate the reactogenicity of a dose of the vaccine in both COVID-19 convalescents whose condition was mild or moderate and those who were asymptomatic with positive PCR, and to evaluate the immunogenicity of a dose of the vaccine in both COVID-19 convalescents whose condition was mild or moderate and those who were asymptomatic with positive PCR.

METHODS

Design and participants

Phase1. Clinical trial phase 1, adaptive, open, and monocentric, carried out in the National Institute of Hematology and Immunology of Havana (Cuba). The adaptive design anticipated the following prospective adaptations:

- Interruption rule because of unacceptable toxicity (if the rate of severe adverse event probability associated with the vaccine was over 0.05).
- Early evaluation of immunogenicity (if the probability of an immune response was over 0.90, a report would be presented to the regulatory agency in order to make progress in the design of the next study).
- Inclusion of other evaluation criteria according to the external data accumulated.

Thirty COVID-19 convalescents were recruited among the ones in Havana who met the selection criteria. The time passed since the hospital discharge or the serological diagnosis until the vaccination was computed.

Participants were distributed among three groups: mild COVID-19 convalescents (N = 11), asymptomatic convalescents (N = 10), both with positive PCR test at the moment of diagnosis and cleared at least two months before the beginning of the study (a safety requisite of the Cuban protocol for convalescent patients), and individuals with subclinical infection detected through community research with IgG specific for SARS-CoV-2, but never confirmed by PCR (N = 9). COVID-19 convalescents of the first two groups had antecedents of hospitalization according to the Cuban protocol. Individuals of the third group were identified during seroepidemiological studies aimed at people without antecedents of COVID-19 clinical manifestations.

All the participants were tested (full clinical record, rapid pregnancy test in women with a fertile age, SARS-CoV-2 PCR tests, blood tests: HIV, serology of hepatitis B and C, full blood recount, tests of kidney and liver function, anti-RBD IgG antibodies, RBD-interaction-blocking antibodies: hACE2, virus-neutralizing test, and cell immunity).

The exclusion criteria were the following: for safety reasons, moderate or severe COVID-19 hospitalization antecedents over the last 2 months, and any severe disease or uncompensated chronic disease, immunodeficiency, antecedents of severe allergy, pregnancy, breast feeding, immunological treatment over the last 30 days; SARS-CoV-2 positive PCR, detection of blocking antibodies of RBD:hACE2 interaction over 60% in a 1/100 serum dilution. The study was registered in the Public Registry of Clinical Trials of Cuba: https://rpcec.sld.cu/en/trials/RPCEC00000349-Sp, included in the WHO Trial Platform of the International Clinical Registry.

Phase II. Clinical Trial phase II, sequential, multi-centric, random, in parallel groups, placebo-controlled, and double-blinded. It was carried out at the National Institute of Hematology and Immunology and the National Center of Sexual Education. Asymptomatic, either mild or moderate COVID-19 convalescents were studied. 450 individuals of both sexes were recruited. Between 19 to 80 years of age, they were chosen among COVID-19 convalescents that met the selection criteria.

The time passed between the hospital discharge and vaccination was registered. All individuals with positive PCR, including the asymptomatic ones, were hospitalized in Cuban hospitals, according to the national regulation. A negative PCR test was required at least two months before the beginning of the study.

The participants were distributed between the experimental group and the control one. The experimental group was vaccinated with a unique dose of SOBERANA®Plus and the control group (placebo) received an excipient of the vaccine. Both the adverse events and the humoral immune response were evaluated.

The study was done sequentially in two phases:

- Phase IIa: open and uncontrolled stage to define the 60-to-80-year-old COVID-19 convalescents' participation in the next phase IIb. This age group would be included in the Phase IIb if the rate of severe adverse events associated with the vaccine were lower than 0.05 and a satisfactory immune response were reached in over 50% of the volunteers.
- Phase IIb: randomized, placebo-controlled, and double-blinded phase. The 19-to-80-year-old COVID-19 convalescents were randomly distributed between two groups: the experimental one, which received the intervention, and the control one, which received the placebo.

All participants underwent screening (full clinical history, rapid pregnancy test in women with a fertile age, de Roche rapid COVID-19 antigen test). The full blood recounts as well as the kidney and liver function tests were only added in phase IIa.

The exclusion criteria were the following: safety reasons, severe COVID-19 history, hospitalization due to COVID-19 over the last two months, any severe disease or uncompensated chronic one, immunodeficiency, severe allergy history, pregnancy, breast feeding, positive COVID-19 test, immunological treatment over the last 30 days. The study was registered in the Public Registry of Clinical Trials of Cuba: https://rpcec.sld.cu/en/trials/RPCEC00000349-Sp, included in the WHO Trial Platform of the International Clinical Registry.

Ethical considerations

The Cuban Ministry of Public Health (MINSAP in Spanish) established a health care program for COVID-19 convalescents. The National Institute of Hematology and Immunology (INHI in Spanish) –clinical site of the trials- and the team of the trial clinical research are included in this health care program.

MINSAP, the Independent Ethical Committee for Studies in Human Beings, in INHI, and the Cuban Regulatory National Agency (State Center for Medication and Medical Device Control, CECMED in Spanish) approved the trial and the procedures:

- Phase I: Authorization Date: 2020/12/30, Reference number: 542/05.017.20B.
- Phase II: Authorization Date: 2021/4/9, Reference number: 110/05.008.21BA.

They were carried out according to Helsinki Declaration and Good Clinical Practice.

An Independent Committee of Data Monitoring (four members specialized in clinical trials and data monitoring, who were independent from both sponsors and researchers) carried out an intermediate analysis of safety data, reactogenicity, and immunogenicity. The final analysis of safety, reactogenicity and immunogenicity was conducted by the statistician in charge of the design and the statistical analysis. All subjects were studied during the intermediate analysis and during the final analysis.

During the recruitment, the researchers provided the potential participants with abundant relevant information, both orally and in writing. All questions were answered. The participation in the study was entirely voluntary. The informed consent of all participants was obtained. During the study, the committees evaluated the risk-benefit relationship of the trial and guaranteed the rights, health, and privacy of volunteers, including the information confidentiality.

Product undergoing evaluation

Vaccine antigen: RBD of SARS-CoV-2 (sequence: 319-541 residues of aminoacids), expressed in CHO cells, purified and characterized as usual. The RBD is dimerized through a disulfide bridge between Cys538-Cys538 links. SOBERANA®Plus vaccine, dose composition (0.5 mL): d-RBD 50 µg, NaCl 4.250 mg, Na2HPO4 0.03 mg, NaH2PO4 0.02 mg, thiomersal 0.05 mg, injection water, aluminum hydroxide gel 1.25 mg, pH 6.0-7.2. The vaccine was manufactured by Finlay Vaccine Institute, in Havana, Cuba, according to the Good Manufacture Practice.

Procedures

Phase I. Blood samples were obtained on days 0 (before vaccination), 7, 14, and 28. Volunteers were closely observed over three hours after vaccination. After vaccination, active monitoring was carried out by health care professionals on days 1 (vaccination), 2, 3, 7, 14, and 28. Participants were instructed to complete a daily registry of both local and systemic adverse reactions, requested over a follow-up period of 28 days.

Over seven days, expected localized reactions were registered. Those reactions were defined by the protocol (pain on the injection spot, heat, reddening, swelling, induration). Systemic symptoms were also registered (general malaise, rash, and a fever, defined as armpit temperature ≥38°C). All other events –especially possible severe adverse events– were registered over the 28 follow-up days.

The severity of the localized and systemic adverse events anticipated, which were defined by the protocol, was classified as mild, moderate, and severe, according to the definition by Brighton Collaboration and the Terminological Criteria Common to Adverse Events version 5.0 [16, 17]. The severity of adverse events not requested was classified as mild (temporary or mild discomfort, without any interference with activity); moderate (mild to moderate activity limitation), and severe (significant activity limitation). The causality of all adverse events was checked and it was classified according to the WHO: causal association inconsistent with immunization, causal association consistent with immunization, indeterminate, unclassifiable. [18]

Phase II. Phase IIa was open, with only one group. Phase IIb was random, with an experimental group and a control one. In the latter, stratified blinded random sampling was used to select the sample among 19-to-80-year-old Cuban citizens, proportionally divided into two age subgroups: 19-59 and 60-80, so as to ensure a proportional representation of each age subgroup according to the national COVID-19 reports.

A 4:1 random distribution was implemented between the experimental group and the placebo-controlled one. The distribution of participants to each group was made by means of simple blinded random sampling using a centralized technology. Each participant received and identification code coinciding with the code of the vial label.

All the personnel that took part in the study, the researchers, the sponsor's staff, and the subjects remained blinded until the end of the study (28 days after administering the vaccine to all the volunteers). All the vials had the same characteristics: R2 flask, unique dose, volume, and pink lid.

Volunteers with COVID-19 antecedents underwent screening; 450 19-to-80-year-old eligible subjects were recruited. In phase IIa, 20 60-to-80-year-old subjects were included. In total, 430 participants were randomly distributed between two groups: the experimental group (vaccine) and the control one (placebo). All the participants were administered a unique dose of either the vaccine or the placebo.

The volunteers were carefully observed over 1h after vaccination. After it, active monitoring was carried out by health care professionals on days 1, 2, 3, 7, 14 (only in phase IIa), and 28. The participants were instructed to complete a daily registry of both the localized and systemic adverse reactions, requested during the 28-day follow-up period. The rest of the procedures were implemented with the same criteria described in phase I.

The basal humoral immune response and that occurred after vaccination in phase I and phase II trial were evaluated by means of the following:

- Quantitative IgG ELISA to detect antibodies against d-RBD, using d-RBD as coating antigen. The trial uses a serum characterized as an internal standard, to which

200 AU/mL were allotted (based on a semi-maximum inhibitory titer of 200 and a conventional virus neutralizing titer of 160). The standard curve was built by making six double dilution series (1:100 a 1:1600). A human anti-γ conjugate was used: peroxidase; the reference curve was built by using the 4-parameter log-logistics function of the Program for the Centers for Disease Control and Prevention. [19]

- Molecular-neutralization-of-the-virus test, based on the blockage mediated by antibodies of the RBD:hACE2 interaction. This test is an in vitro substitute for the neutralization test of living viruses. It uses recombinant mouse RBD-Fc (RBD-Fcm) and the receptor of the host cell hACE2-Fc (ACE2-Fch) as a coating antigen. The human antibodies against RBD can block the RBD-Fcm interaction with ACE2-Fch. The uninhibited RBD-Fcm can join ACE2-Fch and it is recognized by a murine anti-γ monoclonal antibody conjugated with alkaline phosphatase. This inhibition ELISA imitates the virus-host interaction at a molecular level. The inhibition relation of the RBD:hACE2 interaction was calculated to a serum dilution of 1/100 and the inhibitory dilution 50 (mVNT50), which is the serum dilution that inhibits 50 % of the RBD:hACE2 interaction[20].
- Conventional live-virus neutralization test. This assay is the golden standard to determine the efficacy of antibodies against SARS-CoV-2. It is a colorimetric trial based on neutralization by antibodies of the SARS-CoV-2 cytostatic effect in Vero E6 cells. The conventional virus neutralization titers (cVNT) were calculated. [21] A subsample against alpha, beta, and delta variants was evaluated in Hospital Amadeo di Savoia.

In the trial phase I the cell response was studied: after vaccination, the specific RBD T cells producing IFN-γ y TNF-α were quantified by multiparametric intracellular flow cytometry. Briefly, mononuclear cells of peripheral blood (PBMC) were isolated; they were cultivated with the presence of full length recombinant RBD; a solution of brefeldin A; the cells were gathered and first dyed with fluorescent colorant (Invitrogen), and later with anti-CD3 PE/cy7 (SK7) y anti-CD4 PE/cy5 (RPA-T4) extracellular markers. The cells were fixed, made permeable, and dyed with IFN-γ PE (4S.B3) y TNF-α FITC (mAb11). The lymphocytes were obtained in a Gallios cytometer and the data were analyzed with *software* Kaluza version 1.2. [22]

In both studies, the humoral immune response caused by the vaccine was compared with the Cuban Convalescent Serum Panel (CCSP), composed of 68 samples of serum from asymptomatic individuals (25), and the ones recovered from mild/moderate (30) and severe (13) COVID-19, characterized by ELISA, in vitro inhibition trial, and living virus neutralization test.

Variables

The co-primary variables, safety, and reactogenicity were evaluated over the 28 days after vaccination. Safety was measured by the appearance of severe adverse events. The preliminary results were evaluated by the appearance of local and systemic reactions expected and defined by the protocol, as well as unrequested adverse events, on days 7 and 14 after vaccination; the final evaluation was made on day 28 after vaccination. The post-vaccination clinical laboratory tests were compared with the pre-vaccination values.

The secondary result, the vaccine immunogenicity was estimated on days 7, 14, and 28, and was compared with the initial values. The anti-RBD IgG ELISA and the virus molecular neutralization test were carried out on days 0, 7, 14, and 28; the virus conventional neutralization test, on days 0 and 14, and the cellular immunity evaluation, on days 0 and 28.

Phase II. The co-primary variables were safety and immunogenicity. Safety was measured by the appearance of severe adverse events over the 28 days after vaccination. Immunogenicity was evaluated by the virus molecular neutralization test, calculating the satisfactory immune response (mVNT50 ≥250). It was evaluated on days 0, 14 (only in phase IIa), and 28. The clinical laboratory tests run in Phase IIa on day 14 were compared with the pre-vaccination values.

Secondary results: reactogenicity and immunogenicity. Reactogenicity was evaluated daily by the appearance of local and systemic reactions requested and defined by the protocol, over the next 7 days after vaccination, as well as unrequested adverse events, daily over the next 28 days after vaccination.

The vaccine immunogenicity was estimated after vaccination, and compared with the base line: the anti-RBD IgG ELISA and the virus molecular neutralization test were conducted on days 0, 14 (phase IIa), and 28; the seroconversion rate and the RBD:hACE2 interaction inhibition were estimated. The conventional live-virus neutralization test was done on days 0 and 28.

Statistical analysis

Phase I. The calculation of the sample size was based on a severe adverse event rate lower than 5%. Two-sided 95% confidence intervals were calculated for a proportion, with a 0.194 precision.

Safety and reactogenicity are described as frequencies (%). The following values are informed: mean, standard deviation (SD), median, interquartil range, and range for the demographic characteristics and the adverse events. Median, or immunological criteria; geometrical median (GMT) and confidence intervals (CI) of 95%, for mVNT50 and cVNT. The seroconversion rates of the anti-RBD IgG antibodies were calculated (the antibody concentration increased ≥ 4-fold compared with levels prior to immunization) for every subject.

The Spearman correlation range was used to evaluate the relationship among the techniques used to evaluate the immune response. The ROC curve was used to choose the cutoff point most adequate for the humoral tests in relation to the cVNT, and to determine the relationship between sensitivity and specificity for each cutoff point. The Student t test was used or the range test with Wilcoxon sign for the before-after statistical comparison. The statistical analyses were conducted with SPSS version 25.0, STATISTICA version 12.0, R version 3.2.4, EPIDAT version 4.1, Prism GraphPad version 6.0, and WinBugs version 1.4. An alpha significance level of 0.05 was used.

Phase II. The calculation of the sample size for Phase IIa was based on an adverse event rate lower than 5%. Two-sided 95% confidence intervals were calculated for a proportion, with a 0.250 precision. In Phase IIb, the calculation of the sample size was based on a 50% satisfactory immune response; also that the lower limit of the confidence interval for the difference with respect to the control group was higher than 30% and the randomization was 4:1. Two-sided 95% confidence intervals were calculated for the difference between two proportions with an objective width of 0.200. Lastly, 5% of the sample size was added considering the possible abandonment of the study.

Both safety and reactogenicity were described as frequencies (%). The following values were used: mean, standard deviation (SD), median, interquartil range, and range for the demographic characteristics and the adverse events; median for the criteria of immunological evaluation; geometrical mean titers (GMT), and 95% confidence intervals (CI) for mVNT50 and cVNT. The seroconversion rates for the anti-RBD IgG antibodies were calculated.

The Spearman range correlation was used for evaluating the relationships among the techniques used to evaluate the immune response. The Student t test or the Mann-Whitney U test was used for the before-after statistical comparison. A chi-square test was used to determine whether a significant association between two variables exists.

The statistical analyses were carried out with SPSS version 25.0, EPIDAT version 4.1, and Prism GraphPad version 6.0. A 0.05 type I error was used.

RESULTS AND DISCUSSION

Phase I

The mean of the time passed from hospital discharge or serological diagnosis to vaccination was 8 months (ES=1.2), the median was 8.2 months. Mild localized pain was the most frequent adverse event (10%), followed by redness (6.7%). Both were the only local vaccine-associated adverse events found. The solicited systemic reactions were limited to general malaise. Only six subjects (20%) reported adverse events (one reported two local reactions: pain at the injection site and redness). No abnormal laboratory parameters were found related to vaccination.

The frequency of localized and systemic reactions was higher during the first 24h after vaccination; in general, they disappeared over the first three days. The intensity of solicited adverse events was generally mild; only two participants reported a moderate local pain on the vaccination spot. The unsolicited adverse effects were predominantly mild and moderate and they were spontaneously resolved during the follow-up period. The main unsolicited adverse event was high blood pressure, consistent with vaccination in only one case (3.3%). This subject was the only case classified as severe in this clinical trial, but he recovered within the first hour after vaccination. It is important to remember that volunteers with high blood pressure antecedents were included in the study if their blood pressure remained under control during the recruitment.

A significant increase in RBD antibodies was detected on day 7 (median: 146.6 AU/mL). The level of IgG increased on days 14 and 28, with medians of 330.4 and 722.2 AU/mL, respectively. The vaccine brought about a very high increase in the antibody response on day 28. The median values were 14 times higher than those of the CCSP and 21 higher than the level prior to vaccination. Seroconversion was 50% after 7 days. It was 66.67% and 80% after 14 and 28 days, respectively.

The inhibition of the RBD:hACE2 interaction in a 1/100 serum dilution was measured. Twenty-six subjects (86.6%) presented levels of inhibitory antibodies on day 7. These levels are significantly higher than the titers before vaccination and those of the CCSP. On day 7, 23 (76.67%) individuals reached an inhibition index >70 % in a 1/100 serum dilution. On day 14, 26 participants (86.67%) had reacted to vaccination; they reached an inhibition relation of 94% and the median of the titers of inhibitory antibodies was three times higher than that of the CCSP.

In order to evaluate the functionality of antibodies, the mVNT50 value complements the information derived from

the RBD:hACE2 inhibition. High levels or mVNT50 were detected on the 7th post-vaccination day (significantly higher than the titers before vaccination and the CCSP). The GMT of VNT50, on day 28, showed a 103-fold increase compared with the value prior to vaccination (2243,2/21,7) and a 38-fold increase compared with the CCSP value (2243,2/59,3).

The neutralization titers against active SARS-CoV-2 (cVNT) were determined on day 14. The GMT –calculated with all the subjects of the sample– was 234.3, which represents a 5-fold increase compared with the CCSP value (cVNT = 46.4). After vaccination, viral neutralization titers higher than 1:160 were found in 24 (80%) participants.

The cellular immunity was explored by means of anti-CD3 and anti-CD4 monoclonal antibodies to identify lymphocytic sub-populations. The vaccine increased the frequency of specific TNF-α T cells of RBD on day 28. The TNF-α expression of T CD4 + y CD3 + CD4- lymphocytes was significantly higher than that at the beginning (p = 0.00014; IC 95%: 0.43; 1.09 and p=0.00025; IC 95%: 1.19; 4.29, respectively). The frequency of specific CD3 + CD4-T cells of RBD also rose (p = 0.030; IC del 95%: 0.03; 1.09). These are proofs of a cellular immune response triggered by the vaccine.

Only four participants did not increase the level of antibodies after vaccination [a mild COVID-19 convalescent woman, an asymptomatic one; two others, convalescent of subclinical infection (IgG specific to the virus, negative PCR)]. Before vaccination, they had a very low level of anti-RBD antibodies. The two women with mild COVID-19 antecedents and the asymptomatic one increased the CD3+CD4- TNF-α T cells (probably CD8+ T cells) after vaccination. The last one also increased the T CD3+CD4- IFN-γ T cells.

For all the immunological variables analyzed, no differences were found among the three groups. Although the sample size is small, the 95% confidence intervals or the 25-75 percentile ranges of each group overlap, suggesting similarity in the immune response.

Phase II

From April 9 to April 17, 2021, 663 COVID-19 convalescent subjects were recruited for the study, 213 participants were excluded for not meeting the selection criteria and 450 volunteers were added randomly. Twenty 60-to-78-year-old subjects were added to the open uncontrolled phase IIa, and they were administered a unique dose of SOBERANA®Plus vaccine. No severe adverse events were detected and a satisfactory immune response was found in 95% of subjects; therefore, the inclusion of this age group in phase IIb was approved.

Four hundred and thirty 19-to-78-year-old subjects were added randomly 4:1 to the experimental group (N = 344) and the control one (N=86) in phase IIb, and they received a unique dose of the vaccine or the placebo, respectively. Three subjects voluntarily left the experimental group. Eight serum samples were not evaluated due to a major delay in the sample processing: three in the experimental group and five in the control one. All the randomized subjects were included in the safety analysis and immunogenicity was evaluated in most subjects, except those mentioned above.

Between the experimental group and the control one no differences existed in the base line and demographic characteristics. The mean time from hospital discharge to vaccination was 4.5 months (SD = 3.3) in the experimental group and 4.8 months (SD = 3.3) in the control one. Mild COVID-19 predominated in both groups.

The criteria to estimate the sample size were met. The calculation of the sample size in phase IIa was based on a rate of severe adverse events lower than 0.05 and no severe adverse events were notified. The calculation of the sample size in phase IIb was based on a 50% satisfactory immune response, with a lower confidence interval limit for the difference with respect to the control higher than 30%. A satisfactory immune response was found in 80.7% of participants.

Localized pain was the most frequent vaccine-associated adverse events (28.8%). The main solicited systemic reactions were general malaise (6.6%) and headache (4.1%). The frequency of local and systemic reactions was higher over the first 24 h after vaccination; in general, they disappeared over the first 3 days.

Serious vaccine-associated adverse events were not found. The intensity of the solicited adverse events was generally mild; only one subject (0.3%) reported a severe adverse event (headache), but that subject recovered over the first hour after vaccination. Five participants (1.37%) had moderate adverse events: local pain at the vaccination site (3), general malaise (1), and headache (1). The unsolicited adverse events were predominantly mild and they were resolved spontaneously during the follow-up period. No abnormal laboratory parameters related to vaccination were found.

A significant increase of RBD antibodies was detected after vaccination (median: 301.0 U/mL). The median values were 6 times higher than those of the CCSP, 31 times higher than the level prior to vaccination, and 46 times higher than those of the control group (p = 1.9e-27). Seroconversion was 84.4%.

The inhibition of the RBD:hACE2 interaction was measured in a 1/100 serum dilution. On day 28 after vaccination, the levels of the inhibitory antibodies were significantly hi-

gher than the titers before vaccination, and the median of the inhibitory antibody titers (93.5%) was three times higher than that of the CCSP and seven times higher than that of the control group (p = 1.7e-27).

High levels of mVNT50 were detected on 28 post-vaccination day, significantly higher than the pre-vaccination titers, the control group, and the CCSP. The GMT of mVNT50 on day 28 represents a 15-fold increase compared with the CCSP value, a 51-fold increase compared with the pre-vaccination value, and a 45-fold increase compared with the control group (p = 4.7e-33). The mVNT50 ≥ 250 was used to define the satisfactory immune response, and it is the primary result. It was found in the majority of subjects (81%) vaccinated with Soberana®Plus, compared with only 5% in the control group (p = 1.6e-83).

The association between the satisfactory immune response and the classification of the disease, as well as hospital discharge, was demonstrated. A larger number of vaccinated subjects with a satisfactory response in the moderate COVID-19 cases was found, and in the cases with over-4-month-long hospital discharge (p=0.0001). No association with sex, race and age was found.

The live-virus neutralization test was evaluated in 57 subjects: all the subjects of the phase IIa and 37 subjects of the phase IIb. The GMT was 400.3, which represents a 9-fold increase with respect to the CCSP value (cVNT=46.4) and it was 26 times higher than the titers prior to vaccination (p=1.6e-25).

In the Hospital Amadeo di Savoia (Italy) was demonstrated that the vaccine induces neutralizing antibodies against alpha, beta, and delta variants of concern.

There was a good correlation between the cVNT and other variables (coefficients over 0.7), except with RBD:hACE2 inhibition at a 1/100 dilution. The mVNT50 and the cVNT reached the strongest correlation coefficient: 0.889; the correlation was 0.826 for the cVNT and the anti-RBD IgG concentration. There was also a strong correlation between mVNT50 and the anti-RBD IgG concentration (0.934).

Conclusions

SOBERANA®Plus is a very safe vaccine; it is well tolerated, with few adverse events, predominantly local and mild. Only one dose of this vaccine is capable of strongly stimulating the humoral and cellular immunity in individuals with pre-existent natural immunity. It induces neutralizing antibodies against different SARS-CoV-2 variants of concern.

The majority of COVID-19 convalescents is well protected from re-infection after a single dose of SOBERANA®Plus.

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Clinical Trials

Phase I: Public Registry of Clinical Trials of Cuba: https://rpcec.sld.cu/en/trials/RPCEC00000349-Sp.

Phase II: Public Registry of Clinical Trials of Cuba: https://rpcec.sld.cu/en/trials/RPCEC00000366-Sp.

Conflict of interests

The Finlay Vaccine Institute and the Center for Molecular Immunology have filed patent applications related to the use of the vaccine in people with pre-existing immunity to SARS-CoV-2. ROA, YVB, DGR and VVB are investigators at the Finlay Vaccine Institute and BSR is an investigator at the Center for Molecular Immunology, the institutions that manufacture the vaccine. The other authors declare that they have no conflicting interests. No author received fees for this work.

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